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Lisa N. Meihls
USDA-ARS Plant Genetics Research Unit

Man P. Huynh
University of Missouri & Can Tho University

Dalton C. Ludwick
University of Missouri & Can Tho University, daltonludwick@gmail.com

Thomas A. Coudron
USDA-ARS Biological Control of Insects Laboratory, tom.coudron@ars.usda.gov

B. Wade French
North Central Agricultural Research Laboratory

See next page for additional authors

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Research

Comparison of Six Artificial Diets for Western Corn Rootworm Bioassays and Rearing

Lisa N. Meihls,[1,*] Man P. Huynh,[2,3,*] Dalton C. Ludwick,[2,3,*] Thomas A. Coudron,[4] B. Wade French,[5] Kent S. Shelby,[4] Andrea J. Hitchon,[6] Jocelyn L. Smith,[6] Art W. Schaafsma,[6] Adriano E. Pereira,[1] and Bruce E. Hibbard[1,7]

[1]USDA-ARS Plant Genetics Research Unit, 205 Curtis Hall, Columbia, MO 65211, [2]Division of Plant Sciences, University of Missouri, Columbia, MO 65211, [3]Department of Plant Protection, Can Tho University, Can Tho, Vietnam, [4]USDA-ARS Biological Control of Insects Laboratory, 1503 S Providence Road, Columbia, MO 65203, [5]North Central Agricultural Research Laboratory, 2923 Medary Avenue, Brookings, SD 57006, [6]Department of Plant Agriculture, University of Guelph, Ridgetown Campus, 120 Main Street E., Ridgetown, ON NOP 2C0, Canada, and [7]Corresponding author, e-mail: Bruce.Hibbard@ars.usda.gov

*These authors contributed equally.

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Abstract

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is considered the most important maize (*Zea mays* L.) pest in the U.S. Corn Belt. Bioassays testing susceptibility to *Bacillus thuringiensis* Berliner (Bt) and other toxins of corn rootworm larvae often rely on artificial diet formulations. Successful bioassays on artificial diet for corn rootworm have sometimes been challenging because of microbial contamination. Toward the long-term goal of developing a universal artificial diet for western corn rootworm larvae, we compared larval survival, dry weight, and percentage of molt in 10-d bioassays from six current diets of which we were aware. In addition, as part of longer term rearing efforts, we recorded molting over an extended period of development (60 d). Six different artificial diets, including four proprietary industry diets (A, B, C, and D), the first published artificial diet for western corn rootworm (Pleau), and a new diet (WCRMO-1) were evaluated. Western corn rootworm larval survival was above 90% and contamination was 0% on all diets for 10 d. Diet D resulted in the greatest dry weight and percentage molting when compared with the other diets. Although fourth-instar western corn rootworm larvae have not been documented previously (only three instars have been previously documented), as many as 10% of the larvae from Diet B molted into a fourth instar prior to pupating. Overall, significant differences were found among artificial diets currently used to screen western corn rootworm. In order for data from differing toxins to be compared, a single, reliable and high-quality western corn rootworm artificial diet should eventually be chosen by industry, academia, and the public as a standard for bioassays.

Key words: *Diabrotica virgifera virgifera*, artificial diet, diet toxicity assays, resistance monitoring

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is the most significant pest of maize, *Zea mays* L., in the U.S. Corn Belt, with control costs and yield losses exceeding 1 billion dollars (USD) annually (Gray et al. 2009). Heavy larval feeding reduces yield by decreasing the plants ability to uptake water and nutrients and can make root tissue more prone to infection by secondary pathogens such as *Fusarium* species (Palmer and Kommedahl 1969, Kurtz et al. 2010). Root loss associated with larval feeding can also cause plants to lodge, further decreasing yield via unharvested grain (Spike and Tollefson 1988, 1989, 1991). Extremely high adult populations of western corn rootworm prior to anthesis can also decrease yield by clipping ear silks decreasing pollination (Culy et al. 1992). This highly adaptive insect pest has developed resistance to most management tactics including chemical insecticides (Ball and Weekman 1962; Meinke et al. 1998; Pereira et al. 2015, 2017), crop rotation (Levine et al. 2002), and Bt corn (Gassmann et al. 2011, Zukoff et al. 2016, Ludwick et al. 2017).

Bt corn, which expresses insecticidal proteins derived from the bacterium *Bacillus thuringiensis* Berliner, and crop rotation are currently the most effective control measures available for western

corn rootworm. Bt corn, which can contain traits for above- and below-ground herbivores was grown on 92% of U.S. corn acres in 2015 (NASS 2015). There are currently four Bt proteins approved for commercial use by the United States Environmental Protection Agency (EPA) and the Canadian Food Inspection Agency (CFIA) targeting rootworm larvae: Cry3Bb1, Cry34/35Ab1, mCry3A, and eCry3.1Ab. These four proteins, used alone or in combination (pyramids), form the basis of western corn rootworm management in areas of the Midwest where continuous corn planting is prevalent.

The EPA has mandated routine monitoring of insect populations to detect differences in susceptibility attributable to resistance development. A critical component of resistance monitoring, the diet toxicity assay (Siegfried et al. 2005), is dependent on availability of purified protein and an artificial diet for larval feeding. Diet toxicity assays are used in conjunction with other methods of resistance monitoring, such as on-plant assays, to detect decreases in susceptibility (Meihls et al. 2008, Nowatzki et al. 2008, Gassmann et al. 2011, Zukoff et al. 2016). Unfortunately, using differing diets can lead to different results in diet toxicity data (Deans et al. 2017, Ludwick et al. 2018), complicating the comparison of results from different proteins, among different populations, and from different experiments.

Initial research on *Diabrotica* artificial diet formulation and optimization was directed toward the southern corn rootworm, *Diabrotica undecimpunctata howardi* Barber (Coleoptera: Chrysomelidae), as a model species because diet work began prior to availability of the non-diapausing strain of the western corn rootworm (Marrone et al. 1985). An attempt to optimize a diet for rearing of western corn rootworm occurred in 2002 (Pleau et al. 2002). Subsequently, modified, proprietary diet formulations have been developed by each of the major maize seed companies. Evaluating rootworm toxins on differing diets does not allow direct comparisons between proteins because of differences in artificial diet formulations. In fact, nutrition may have a significant effect on the toxicity of Bt in western corn rootworm as has been shown for lepidopteran species (Janmaat and Myers 2005, Bird and Akhurst 2007, Raymond et al. 2007, Blanco et al. 2009, Orpet et al. 2015). In the cabbage looper (*Trichoplusia ni* Hübner) previously unexposed insects fed Cry1Ac-incorporated diet were less susceptible on diets containing a low protein:carbohydrate (35:65) ratio than diet containing a high protein:carbohydrate ratio (90:10) (Orpet et al. 2015). If nutrition similarly affects coleopteran response to toxins, then differences in the artificial diets between individual companies will make direct comparisons between assays problematic, and may not provide an accurate phenotypic picture of the test populations as it relates to susceptibility to toxins.

A single optimized artificial diet is necessary to facilitate long-term rearing, accelerate novel product discovery, and standardize corn rootworm resistance monitoring assays. We identified four unique proprietary artificial diets in use that were included in this study. In addition to the four proprietary artificial diets, an older publicly available western corn rootworm diet (Pleau et al. 2002) and a recently optimized artificial diet (Huynh et al. 2017), referred hereafter as WCRMO-1, were also included in this study. The objective of this study was to evaluate and compare corn rootworm larval survival, larval dry weight, and molting, in 10 d assays on all the diet formulations. In addition, we carried some experiments beyond 10 d and discovered an unusual extra molt, to fourth instar.

Materials and Methods

Insects and Egg Treatment

A laboratory maintained non-diapausing western corn rootworm population was used in all assays. Eggs were obtained from the primary non-diapausing colony at the North Central Agricultural Research Laboratory in Brookings, SD (Branson 1976, Kim et al. 2007). Egg plates were incubated at 25°C in complete darkness until hatching larvae were observed. When approximately 50 hatching larvae were observed, soil was removed by rinsing egg plate contents through a 250-µm, #60-mesh sieve (Hogentogler & Co. Inc., Columbia, MD), which retained the eggs. Eggs were washed from the sieve into a 100-ml beaker using a gentle stream of warm water and were triple rinsed with tap water to float away soil debris, larvae, and egg cases. All products used in subsequent egg handling were either purchased sterile or sterilized via exposure to 10% bleach (The Clorox Company, Oakland, CA; Clorox Regular Bleach) solution for 15 min followed by exposure to UV-C for 10 min in a biological safety cabinet.

Eggs free of debris and in a small volume of water were surface sterilized prior to use in assays (Pleau et al. 2002). Eggs were first exposed to 10–20 ml of undiluted Lysol (Reckitt Benckiser, LLC, Parsippany, NJ; Clean & Fresh Multi-Surface Cleaner) (exact volume depended on the volume of eggs being sterilized) for 3 min. The beaker was swirled gently several times to ensure complete exposure of egg surfaces to the Lysol. After 3 min, the supernatant was removed, and eggs were triple-rinsed in 10–20 ml of purified water (Milli Q). Next, eggs were rinsed with 10–20 ml of Formalin-10% formaldehyde (Sigma Aldrich, St. Louis, MO; HT501128) with a drop of Ivory (Proctor & Gamble, Cincinnati, OH; Ivory Dish Detergent) soap added to the beaker and swirled for 3 min to expose all egg surfaces to the disinfectant. The supernatant was again removed, and eggs were triple-rinsed in 10–20 ml of distilled water leaving enough water to allow eggs to be collected in a 1-ml disposable pipette (Fisher Scientific, Pittsburg, PA; 13-711-9a). Eggs were dispensed from the pipette in a uniform layer across the bottom of a UV-C sterilized coffee filter (Rockline Industries, Sheboygan, WI; Pure Brew 8–12 cup). Coffee filters were first blotted to remove excess water, then placed inside bleached 473.2 ml Solo deli cups fitted with plastic lids (Solo Cup Company, Lake Forest, IL; LG8RB-0090 and DM16R-0090). For ventilation, several holes were made in the lid using a number one insect pin. Eggs were placed in an incubator at 25°C in complete darkness until hatching larvae were observed (usually within 24 h). Coffee filters were transferred daily to new deli cups with new lids until experiments were completed or eggs ceased to hatch.

Larval Diet Bioassays

All products used in larval handling were either purchased sterile or sterilized via exposure to 10% bleach solution for 15 min followed by exposure to UV-C for 10 min in a biological safety cabinet. Diet plates with 96 wells from individual companies were received biweekly prior to use in bioassays according to the company specifications. Diet was wrapped in plastic and stored at 4°C in complete darkness until used in bioassays. Bioassays were conducted using neonate larvae (<24 h after hatching). A single neonate larva was placed in each of the 96 wells for each plate using a fine tipped paintbrush. Wells were sealed using a pressure sensitive silicon adhesive 96-well cover (Excel Scientific, Inc., Victorville, CA; TSS-RTQ-100) and a tiny hole created with an insect pin (size one) to facilitate air exchange. Except when recording data, plates were stored at 25°C in complete darkness for the duration of the assay. Each diet was replicated six times on separate diet plates. Within each replication, there were 24 pseudo-replications of individual larvae/plate. Larval mortality, larval molting, survival, and evidence of diet contamination were recorded daily for 10 d; after which, live larvae from uncontaminated wells were collected into 70% ethanol, dried in a

drying oven for 48 h (Blue M Therm Dry Bacteriological Incubator, Model #602752), and weighed (Sartorius Lab Instruments GmbH & Co. KG, Goettingen, Germany; MSU6.6S-000-DM). Up to 24 larvae recovered from each replication were pooled, weighed, and average weight for that replication calculated by dividing by the number of larvae.

Diet Preparation

Proprietary diets from the various collaborating companies were prepared at their laboratories and received fresh at the beginning of each experiment. The WCRMO-1 and Pleau et al. (2002) diets were prepared at a similar time as the proprietary diets to standardize the age of all diets. Preparation of the WCRMO-1 diet was similar to that described in Pleau et al. (2002), with modifications (Huynh et al. 2017). All diet preparation for the Pleau et al. (2002) and WCRMO-1 diets, except for the initial weighing of ingredients and the microwaving of the agar, were performed inside a biological safety cabinet that had been surface sterilized using UV-C exposure for 10 min. All materials required for preparing the artificial diet were first sterilized by soaking in 10% bleach solution for 15 min, air dried, and then surface sterilized via exposure to UV-C for 10 min while in the laminar flow hood. Beakers and the blender jar were heated to 50°C immediately before diet preparation.

Briefly, water and agar were mixed in a 400-ml beaker and microwaved on high until clear. The boiling agar solution was transferred to a laminar flow hood and allowed to cool to 50°C before being added to the blender (Hamilton Beach Brands, Inc., Southern Pines, NC; 51101b). Once the agar solution reached 50°C, dry diet ingredients were added. The diet was then blended intermittently, enough to incorporate all of the ingredients but not excessively to avoid forming air bubbles. When the diet appeared uniformly mixed, liquid ingredients were then added. KOH (GE Healthcare Bio-Sciences, Pittsburg, PA; Whatman 09-876-18) was added as needed to adjust the pH of the diet to 9.0, which was clearly documented as significantly better for larval mass gain than a pH of 5 and also better than a pH of 7.5 (Pleau et al. 2002). The blended diet was transferred to a low-sided beaker (Cole-Parmer Instrument Company, LLC., Vernon Hills, IL; 3140, 125 × 65) containing a stir bar and placed on a hot plate to maintain a constant temperature (50°C) when dispensing. Using a multichannel pipette (Eppendorf, Hamburg, Germany; Eppendorf Repeater Plus/8 Pipette, 022264109), 200 µl of diet was dispensed into each well. Poured diet plates were allowed to cool in the laminar flow hood for ~30 min before being covered with the plate lid, wrapped in plastic wrap (Glad ClingWrap, The Glad Products Company, Oakland, CA), and stored at 4°C.

Extended Bioassays

Bioassays originally aimed at adult emergence were similar to larval diet bioassays, except that larvae were transferred to a new diet plate every 10 d initially and every 7 d as larvae grew larger. This was performed to ensure that diet nutrient content did not change over time with exposure to 25°C. Following sterilization procedures of transfer equipment, larvae were removed from initial 96-well plates and transferred to new 96-well plates in a laminar flow hood. Larvae that did not molt to the second instar by 20 d were removed from the study. The same occurred for larvae that did not reach the third instar by 40 d, and pupae by 60 d. Larvae in the second and third instars were transferred with soft forceps. Pre-pupae and pupae were not transferred from the plate in which these stages were observed. Wells were sealed as described previously and plates returned to the chamber at 25°C in complete darkness for the duration of the assay. This procedure was repeated until larvae died or emerged as adults. Larvae in contaminated wells were not transferred to the new diet plate. Larval mortality, larval molting, pupation, adult eclosion, and evidence of diet contamination were recorded daily or every 2 d for the duration of the assay.

Statistical Analysis

The 10-d bioassays were set up and analyzed as a randomized complete block experiment. All data for 10-d bioassays were analyzed with analysis of variance (ANOVA) in a generalized linear mixed model (PROC GLIMMIX) in SAS 9.4 (SAS Institute, Cary, NC). Diet was the fixed effect and replication was the random variable. Differences between diets were determined using Fisher's LSD.

Survival was calculated by dividing the number of survivors by the initial number of individuals. Survival values were analyzed with a binomial distribution with a logit function. Average weight was calculated by dividing the total dry weight of survivors by the initial number. Average weight data had a normal distribution. Two diets (Diets B and C) did not produce second-instar larvae, so they were removed from the analysis of molting data. Molting data were calculated by dividing the number of second-instar larvae by the number of survivors. The molting analysis used a binomial distribution with a logit function.

The extended bioassay was set up and analyzed as a randomized complete block. All data for extended bioassays were analyzed with ANOVA in a generalized linear mixed model (PROC GLIMMIX) in SAS 9.4 with a beta distribution and a logit function (SAS Institute, Cary, NC). Prior to calculating the ratio for analysis, we added 1 to both the number of larvae that reached fourth instar and the initial number of larvae since the log of zero cannot be calculated. Diet was the fixed effect and replication was the random variable. Differences between diets were determined using Fisher's LSD.

Results

All six diets had 0% contamination for the full 10 d of the first experiment. Larval survivorship to 10 d ranged from 91.9% for the Pleau et al. (2002) diet to 100% for Diet D (Fig. 1). Overall, diet had a marginal effect on survival percentage (F = 2.42; df = 5, 30; P = 0.0588), but a highly significant effect on average larval dry weight (F = 69.49; df = 5, 30; P < 0.0001) and larval molting from first to second instar within 10 d (F =27.16; df = 3, 27; P < 0.0001). There was no significant difference between the WCRMO-1 diet and any other diet in terms of percent survival (Fig. 1). The Pleau et al. (2002) diet and Diet C had significantly lower survivorship than Diet A and Diet D according to Fisher's LSD on the marginal data set (Fig. 1). Average larval dry weight after 10 d of feeding on each diet varied significantly (Fig. 2). Average larval dry weight on Diet D was more than 0.2 mg, while average dry weight on Diet C was only 0.017 mg, nearly a 12-fold difference (Fig. 2). Differences between diets were all significant except between the Pleau et al. (2002) diet and the WCR-MO1 diet. No larvae molted to second instar within 10 d on Diets B and C, so these were excluded from the statistical analysis for percent molting. Significantly more larvae molted when reared on Diet D than on the other diets, and significantly more larvae molted on the WCRMO-1 diet and Pleau et al. (2002) diet than on diet A (Fig. 3).

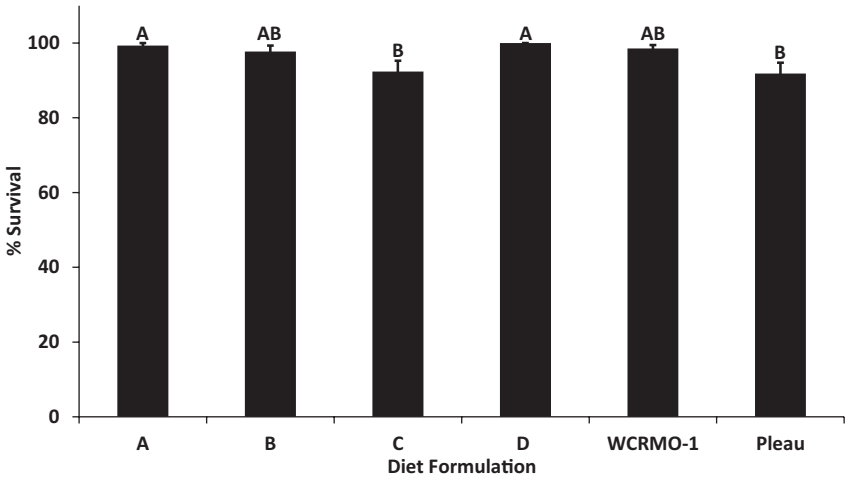

Fig. 1. Average (±SE) percentage survival of western corn rootworm larvae after neonate larvae were fed for 10 d in diet assays with different diet formulations. Bars followed by different letters are significantly different in least square means using PROC GLIMMIX in SAS 9.4 at $\alpha = 0.05$.

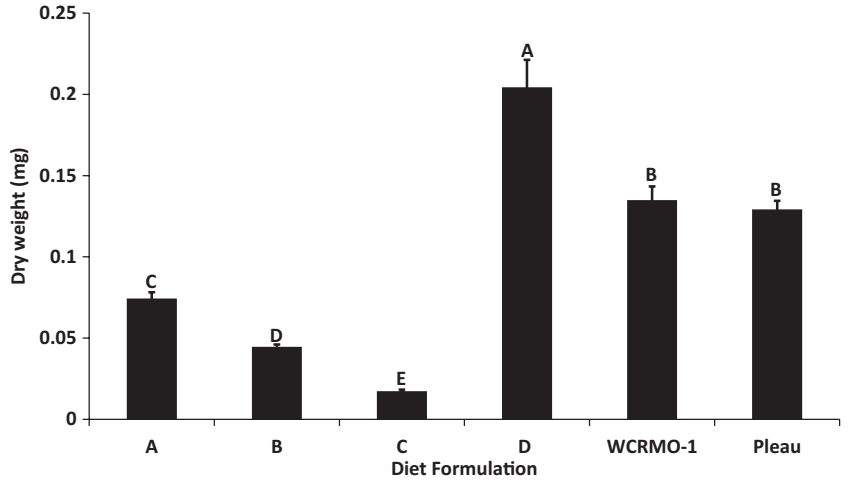

Fig. 2. Average (±SE) dry weight (mg) of western corn rootworm larvae recovered after feeding 10 d on different diet formulations. Bars followed by different letters are significantly different in least square means using PROC GLIMMIX in SAS 9.4 at $\alpha = 0.05$.

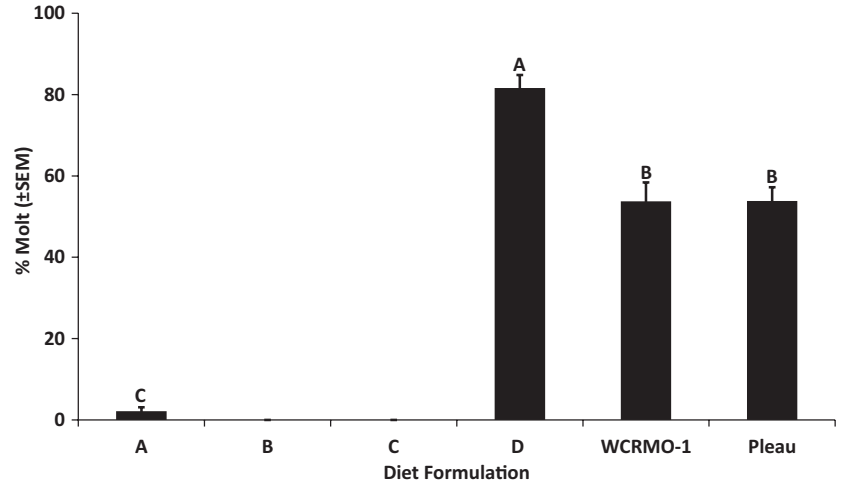

Fig. 3. Average percentage of western corn rootworm that molted to the second instar after feeding for 10 d on different diet formulations. Bars followed by different letters are significantly different in least square means using PROC GLIMMIX in SAS 9.4 at $\alpha = 0.05$.

Extended Bioassays

During the longer bioassays, it was noticed that a percentage of the western corn rootworm third-instar larvae molted to supernumerary fourth instar. There were significantly more fourth-instar larvae on Diet B compared with WCRMO-1 and Pleau et al. (2002) ($F = 4.39$; df = 5, 24; $P = 0.0056$), whereas Diet A and Diet C exhibited the lowest rates of fourth-instar larvae (Fig. 4). Diet C was excluded from the statistical analysis because no third instars developed on this diet formulation.

Discussion

In the original baseline susceptibility manuscript for Cry3Bb1 (Siegfried et al. 2005), mortality and individual larval weights were recorded 4–7 d after adding neonate larvae to the diet plate. Much of the reason for the variable endpoint had to do with contamination, which has been a major issue in these types of assays (Pereira et al. 2016). Improved bioassay techniques have helped overcome some of the contamination issues in western corn rootworm artificial diet assays (Magalhães et al. 2007), but issues have remained (Pereira et al. 2016). In this study, we observed zero percent contamination for all six diets for at least 10 d, which indicates that diet components are not the cause of contamination when strictly adhering to clean laboratory practices. All products used in larval handling were either purchased sterile or sterilized (UV/ethanol/10% bleach). In addition, nearly all procedures were performed in a biosafety cabinet, and eggs were used within 3 d of sterilization. Sterilization of the eggs with Lysol and 10% formaldehyde seems to be one of the key factors in preventing contamination in diet toxicity assays for the western corn rootworm. Similarly low contamination rates were seen in Huynh et al. (2017) and in Ludwick et al. (2018).

Our results demonstrate inherent differences in nutrition across diets currently used to rear western corn rootworm larvae. Some of these differences were small such as survival (Fig. 1), while others, such as weight, were much more apparent (Fig. 2). Nutritional differences have been determined to be a factor in Bt susceptibility in lepidopterans (Deans et al. 2017) and these differences could likely impact the interpretations of diet toxicity assays for western corn rootworm as well. Inadequate nutrition could lead a researcher to believe a product causes developmental impacts. For instance, two diets failed to produce second-instar larvae (Fig. 3). Yet, even the best diets appear to be inferior to maize (Huynh 2018), so there is a considerable room for improvement.

The WCRMO-1 diet has been fully evaluated for its nutritional content (Huynh et al. 2017) and its compatibility with the Bt proteins currently marketed for control of western corn rootworm (Ludwick et al. 2018). While Diet D did have greater weight and molting rates, this particular diet is proprietary and not available for researchers without a Material Transfer Agreement. Furthermore, the exact composition of Diet D is not publicly available. The WCRMO-1 diet outperformed Diets A–C for dry weight and molting (Figs. 2 and 3). As these proprietary diets are the diets on which diet toxicity assays to assess Bt susceptibility are conducted, we have confidence in the WCRMO-1 diet to be a publicly available formulation that provides a reasonable source for western corn rootworm nutrition and Bt resistance monitoring. Our group will continue to evaluate new diets modified after Huynh et al. (2017). We plan to continue evaluating prospective improvements toward equaling Diet D for molting, larval weight, and survivorship (now done Huynh 2018) and toward equal performance on maize, so that complete western corn rootworm rearing until adulthood in artificial diet is possible.

The supernumerary larval growth to the fourth instar reported here in western corn rootworm is not uncommon in other insects also reared on artificial diets. Many other research studies have reported an additional larval instar before pupation due to adverse conditions (Esperk et al. 2007; Esperk and Tammaru 2010). For example, some insect species tend to have an extra instar, especially when reared individually (Esperk et al. 2007). However, extra molts in western corn rootworm have not been noted previously to our knowledge. Since extra molts have not been noticed on maize, this may indicate suboptimal nutrition provided by the artificial diets, or adverse abiotic factors from the containers used. Several theories attempt to explain this particular behavior in insects. Poor nutrition in the diet has been considered as the main reason for supernumerary instars. Insect larvae may compensate for poor nutrition by increased feeding in an attempt to obtain the missing nutrients and to complete development. Diet B presented the highest level of fourth instars, which suggests that it might be of poor quality when compared with the other diets except Diet C, which did not even produce third-instar larvae.

For the relevant comparison, larval weight of western corn rootworm on maize under greenhouse conditions averaged between

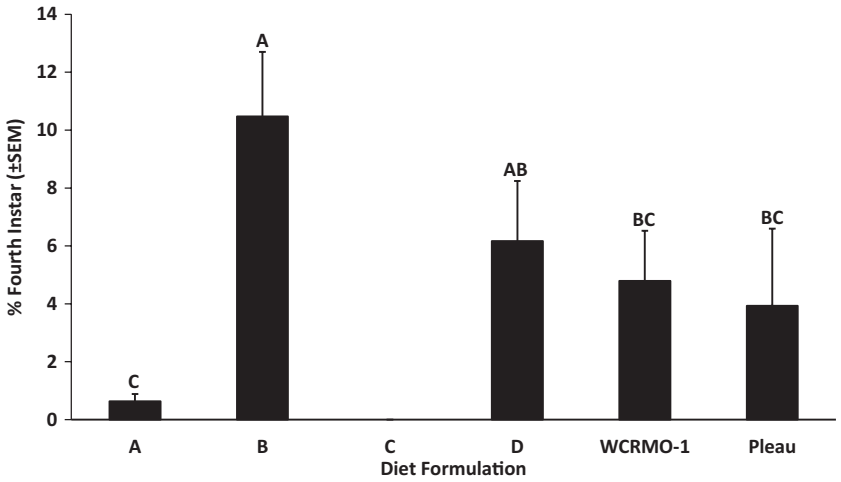

Fig. 4. Average percentage of western corn rootworm larvae that molted to fourth instar larvae in each western corn rootworm larval diet formulation in extended diet assays. Bars followed by different letters are significantly different in least square means using PROC GLIMMIX in SAS 9.4 at $\alpha = 0.05$.

0.18 mg (Oyediran et al. 2004) and 0.26 mg (Clark and Hibbard 2004) when reared on corn for 10 d. Diet D was the only diet to exhibit average larval weight within this range (Fig. 2), yet fourth-instar larvae were present on this formulation (Fig. 4). Overall, our results clearly demonstrate that there are large differences between the artificial diets currently utilized for corn rootworm resistance monitoring. A standardized, available western corn rootworm artificial larval diet would enable researchers to produce comparable results across different laboratories and with differing toxins.

Acknowledgments

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